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Synthesis and Characterization of Polymers Produced by Horseradish Peroxidase in Dioxane

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SYNOPSIS

Polymers were synthesized from substituted phenolic and aromatic amine compounds with hydrogen peroxide as the source of an oxidizing agent and horseradish peroxidase enzyme as the catalyst. The polymerization reaction was carried out in a monophasic organic solvent with small amounts of water at room temperature. Conditions for the synthesis of polymers with respect to reaction time and yield were studied with a number of monomers at different concentrations and in solvents with different buffers with pH range of 5.0–7.5. Physical and chemical properties of these homo- and copolymers were determined with respect to melting point, solubility, elemental analysis, molecular weight distribution, infrared absorption (including FTIR), solid-state ^{13}C nuclear magnetic resonance, thermal gravimetric analysis, and differential scanning calorimetry. The enzyme catalyzed reactions produced polymers of molecular weight greater than 400,000 which were further fractionated by differential solubility in solvent mixtures and the molecular weight distribution of the polymer fractions were determined. In general, the polymers synthesized have low solubilities, high melting points, and some degree of branching.

INTRODUCTION

Phenolic polymers are widely used in a number of applications and phenol-formaldehyde resins that constitute the major part of the phenolic polymers, are synthesized from formaldehyde and phenolic compounds.[1] Oxidative coupling reactions of alkylphenols in the presence of catalysts are also used for the synthesis of phenolic polymers poly(phenylene ether) and poly(*p*-phenylene).[2] Because of the hazardous nature of the starting materials, and extremes of temperature, pressure, and pH required for the synthesis of these polymers, concerns have been expressed about this synthetic approach. The enzymatic approach for the synthesis of phenolic polymers has been described earlier.[3] In this article we have expanded the scope of these reactions and presented the first systematic and detailed characterization of these polymers produced by enzyme catalyzed reaction using different phenols and aromatic amines. These enzyme-catalyzed polymerizations were carried out at room temperature and pressure, and at pH from 5.0 to 7.5.

Enzyme-catalyzed reactions are generally carried out in aqueous media for optimal catalytic activity. In aqueous solutions the hydrophilic residues of the enzyme are oriented on the surface of the molecule in contact with water for catalytic activity, and the hydrophobic residues of the enzyme are buried in the interior of the enzyme molecule. A variety of enzymes have been shown to be catalytically active,[4-11] with increased enzyme stability,[8] in the presence of organic solvents with small amounts of water. High-resolution solid-state NMR studies with alpha lytic protease have shown no major change occurs in the conformation of the enzyme when the dry enzyme is suspended in anhydrous acetone or octane.[12] The level of hydration of the enzyme, the pH from which the enzyme was lyophilized, and the solvent and substrate hydrophobicity and dielectric constants are some of the factors that determine the activity of the enzyme in an organic solvent.

Enzyme catalyzed reactions in nonaqueous solvent systems offer benefits such as: (1) increased

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solubility of substrates in solvents and hence more efficient catalysis, (2) reduction in the number of undesirable side reactions, (3) shifting of the reaction equilibrium in favor of synthesis over hydrolysis, (4) ease of product separation from the reaction mixture, (5) enhanced thermal stability of enzymes and possible biocatalysis reactions at a higher temperature, and (6) ease of enzyme recovery.[4] Some of these changes in enzyme catalyzed reactions in nonaqueous solvents have been utilized for the enantioselective synthesis of peptides/monoesters,[5,9,13] lignin depolymerization,[7] regioselective interesterification of lipids,[11] polymerization of phenols,[3] transesterification by lipase at high temperatures,[14] synthesis of optically active cyanohydrins,[15] and oxidation of steroids.[16]

This article will discuss the synthesis of homopolymers and copolymers catalyzed by horseradish peroxidase reactions carried out in a water-miscible organic solvent system, and the characterization of these polymers. Other solvent systems for biocatalysis reactions, anhydrous organic solvents with less than 1% water, biphasic systems with water immiscible solvent and water, reverse micelles dispersed in nonpolar solvents, and supercritical fluids have been reviewed in literature.[4,8,17,18]

EXPERIMENTAL

Materials

Horseradish peroxidase (Type II, 150–200 units/mg solid), hydrogen peroxide, and buffers were purchased from Sigma Chemical Company, St. Louis, MO. Phenolic and aromatic amine compounds were obtained from Aldrich Chemical Company, Milwaukee, WI. Solvents used were high performance liquid chromatography (HPLC) grade and purchased from Caledon Laboratories, Ontario, Canada.

Methods

The synthesis of polymers was carried out in a water-miscible solvent, dioxane, with a buffer at room temperature. The ratio of the buffer to the solvent varied depending upon the monomer being used for the synthesis of polymers. Polymer synthesis was evaluated with three different buffers: acetate @ pH 5.0, phosphate @ pH 7.0, and HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) @ pH 7.5. The monomer was dissolved in the solvent, and the horseradish peroxidase (solubilized in the buffer being used) was added slowly to the solvent (with the monomer) with gentle mixing. The catalytic reaction was initiated by the addition of hydrogen peroxide. The final reaction mixture contained 0.1 *M* monomer, 0.5 mg per mL horseradish peroxidase, and 0.1 *M* hydrogen peroxide in a water-miscible solvent containing a buffer. The synthesis was terminated after 2–24 h, depending upon the rate of formation of polymer. Polymer was isolated by centrifugation at 5000 × g at room temperature for 10 min, and washed with water followed by the solvent used in the reaction to remove residual buffer, horseradish peroxidase, unreacted monomers and low molecular weight polymer. Blank runs without enzyme were carried out to determine non-enzymatic formation of polymer. No polymer synthesis was observed in the blank runs.

The synthesized polymers were characterized as follows: melting point was determined by a bench top capillary point apparatus (Thomas Hoover, Arthur H. Thomas Co., Philadelphia, PA), solubility was evaluated at the concentration of 100 mg of polymer per 100 mL of the solvent, elemental analysis of copolymers containing nitrogen functional groups was carried out at 980°C in an atmosphere of oxygen with an Automatic Control Equipment Analyzer (Model 240XA, Control Equipment Corp, Lowell, MA), thermal properties were determined by a Thermal Gravimetric Analyzer (TGA) (Model 951 Du Pont, Wilmington, DE) and a Differential Scanning Calorimetry (DSC) (Model 910, Du Pont). For thermal analyses (TGA and DSC) the flow rate of nitrogen gas was 70 mL/min, the sample size was 2–10 mg, and the temperature gradient was 5 or 10°C/min. The data were analyzed by Thermal Analyzer (Model 1090, Du Pont) equipped with TGA Analysis V1.0 program and Interactive DSC V2.0 programs (Du Pont). The molecular weight of the polymers were determined by column chromatography using TSK-GEL Toyopearl packing (HW-55 F, Supelco, Bellefonte, PA) with a dimethylformamide : methanol (DMF : MeOH) solvent mixture in the ratio of 4 : 1 (V/V) as the eluant. Other column packings studied included μ Styragel (Waters Div., Millipore, Bedford, MA) and LH-60 (Pharmacia, Piscataway, NJ). The polymer was first solubilized in the solvent mixture at a concentration of 0.1% (W/V), 1.0 mL of the polymer solution was applied to the column, and 1.0 mL fractions were collected and assayed for optical density at 270 nm. The fractions were also scanned from 190 to 500 nm to determine absorption spectra. Polystyrene standards (Waters Div., Millipore Corp) were used as the reference material to calibrate the column.

To isolate and study the properties of the different

fractions of the polymer synthesized, the polymer fractions were separated by differential solubility in solvent mixtures of DMF : MeOH at different ratios. The molecular weights of these polymer fractions were determined as described above. The poly(*p*-phenylphenol) polymer was solubilized at a concentration of 0.1% (W/V) in DMF : MeOH at a ratio of 4 : 1, and the ratio of the solvent mixture was then changed to 8 : 5 by adding more methyl alcohol. The precipitate that formed in the new solvent mixture was separated by centrifugation (at 5000 × g) and washed twice in the same solvent mixture. The precipitate was resolubilized in the DMF : MeOH (4 : 1) solvent mixture at a concentration of 0.1% (W/V) and applied onto the Toyopearl TSK-GEL column to determine the molecular weight distributions of the fraction precipitated in the 8 : 5 solvent mixture. An aliquot of the soluble fraction from the 8 : 5 fractionation was also applied to the Toyopearl TSK-GEL column to determine the molecular weight distribution of the polymer in the soluble fraction. The 8 : 5 soluble fraction was further fractionated by adding more methyl alcohol to give a solvent ratio 8 : 9 of DMF : MeOH. The precipitate formed was separated, washed twice, solubilized in DMF : MeOH (4 : 1) solvent mixture [at 0.1% (W/V) concentration], and evaluated by column chromatography as before.

Fourier transform infrared spectroscopy was performed on a Nicolet, Model # 20SXB (Madison, WI) with a continuous scan Michelson interferometer, wave number accuracy 0.001 cm^{-1} throughout the spectral range controlled by HeNe laser, multi-layer germanium coated KBr, and high-sensitivity, cooled Mercury Cadmium Telluride detector. Spectroscopic properties of the polymers were evaluated using potassium bromide pellets at a concentration of 1.0% (W/W) polymer.

To deactivate the phenolic groups present in poly(*p*-phenylphenol) and thereby decrease the degree of crosslinking and/or branching observed during heating of the polymer, the enzymatically synthesized poly(*p*-phenylphenol) was treated with epichlorohydrin in alkaline solution. The polymer (100 mg) was suspended in 1.0 mL NaOH (0.25*N*), the suspension was stirred for 10 min, and 10 μL of epichlorohydrin was added to this mixture. The stirring of the reaction mixture continued until 10 min, when the reaction was quenched by the addition of 20 mL distilled water. The unreacted epichlorohydrin and sodium hydroxide were removed by repeated washings in water followed by washing with a solvent mixture of dioxane : water (85 : 15, V/V) and the polymer was air dried.

^{13}C solid-state NMR of polymers was carried out at 68 MHz, with cross-polarization magic-angle sample spinning (CP/MASS) (with a spin speed of 3.8–4.3 kHz) with SDS 270 (Spectral Data Service, Inc., Champaign, IL). Chemical shifts were calibrated through the external standard of tetramethyl silane. A deconvolution program was used for the partition and identification of the NMR peaks. The proton NMR spectrum of polybenzidine in a solution of dimethyl-d_6 sulfoxide (DMSO-d_6) was determined at 360 MHz (Nicolet, Model # 360, Madison, WI). NMR studies with polybenzidine in DMSO(d6) also were carried out after D_2O exchange, to identify the amine protons present in the polymer.

RESULTS AND DISCUSSION

Table I lists the monomers evaluated for the synthesis of homopolymers and copolymers with horseradish peroxidase in a dioxane solvent buffer mixture (85 : 15) at pH values of 5.6, 7.0, and 7.5. Copolymers were prepared from a mixture of substituted phenolic and aromatic amine monomers.

The monomers are arranged according to their functional groups, phenolic, aromatic amine, or both. Although most of these substrates reacted, as evidenced by color formation under the experimental conditions, only some of monomers produced a polymeric precipitate for subsequent chemical and physical characterization. In general, the polymers prepared from phenolic monomers exhibited high melting range greater than 300°C, while the melting ranges of the corresponding copolymers synthesized with phenolic and aromatic amine functional groups had melting ranges lower than 300°C. Ring-substituted phenolic and aromatic amine monomers were evaluated to reduce branching reactions from the aromatic rings during the enzyme-catalyzed polymerization. However, the reactions were too slow to produce any recoverable polymer.

Table II shows the effect of solvent to buffer ratio and pH of the buffer on the melting point and yield of the polymer synthesized from *p*-phenylphenol. Only low molecular weight oligomers, mostly dimers and trimers, were formed when the reaction was carried out in buffer without dioxane. In general, the polymer yield decreased with an increase in solvent concentration in the reaction mixture over the range studied, while the melting point of the polymer increased with the increase in solvent concentration. The results indicate that the yield and melting point range of the polymer can be controlled by adjusting the pH of the buffer and the concentration of solvent

Table I. Monomers Evaluated for the Synthesis of Polymers

Phenolic Functional Group	Amine Functional Group	Mixed Functional Groups
Phenol	Aniline	8-Hydroxyquinoline
Anisole	Benzidine	Isoquinoline
Cresols (*o*, *m* & *p*)[a]	3-Phenylenediamine	Tyrosine
1,2-Benzenediol[a]	Phenylethylamine	4-Phenylazo phenol
2-Hydroxybenzylalcohol[a]		4-(2-Pyridylazo)resorcinol
2-Methoxyphenol[a]		2-Methyl 8-quinolinol
3-Methoxyphenol[a]		4-Amino *m*-cresol
3,4-Dimethylphenol[a]		
4-Phenylphenol[a]		
3-Phenylphenol[a]		
4-Phenoxyphenol[a]		
3-(3-Phenoxyphenoxy)phenol[a]		
Diethylstilbesterol		
1-Hydroxynaphthalene		
2-Hydroxynaphthalene		
1,3-Dihydroxynaphthalene		
1,5-Dihydroxynaphthalene		
Hematein		

[a] Substitued phenolic compounds used to control the reaction and decrease branching and crosslinking in the polymer.

in the reaction mixture. Similar results were reported by Dordick et al.[3] for the synthesis of poly(*p*-phenylphenol) using horseradish peroxidase in dioxane solvent with acetate buffer.

Table III shows the elemental analysis of the copolymers prior to fractionation. The calculated percentages of carbon, hydrogen, and nitrogen of the monomer mixtures used in the synthesis of copolymers are given in parentheses. The results indicate that different monomers were incorporated into the copolymers and the ratio of incorporation varied with the monomers used in the reaction mixture.

Table II. Effect of Solvent/Buffer Ratios on Yield and Melting Range of Poly(*p*-Phenylphenol)

Solvent/Buffer[a]	Yield (%)	Melting Range (°C)
pH 7.0		
75% Dioxane/25% PO_4	73	250–255
80% Dioxane/20% PO_4	49	218–257
90% Dioxane/10% PO_4[b]	24	260–292
95% Dioxane/5% PO_4[b]	2.2	240–300
pH7.5		
75% Dioxane/25% HEPES	71	230–245
80% Dioxane/20% HEPES	93	215–250
90% Dioxane/10% HEPES	29	290–> 300
95% Dioxane/5% HEPES	Negligible	—

[a] The reaction time was 2 h except with two solvent systems given below. Other reaction conditions are given in the text.
[b] The reaction time was 18 h at room temperature.

Thermal properties of a number of polymers were determined by TGA and DSC, and thermograms of two of these polymers, poly(*p*-phenylphenol) and polybenzidine, are illustrated in Figures 1 and 2. In general, the TGA analyses (Fig. 1) indicated that a significant amount of material remains after heating the polymer to 1000°C in an atmosphere of nitrogen gas.

TGA of poly(*p*-phenylphenol) indicated about 40% of the polymer was lost either by evaporation and/or by degradation on heating the polymer to 600°C in a stream of nitrogen gas [Fig. 1(A)]. Thermal analysis of poly(*p*-phenylphenol) treated with epichlorohydrin indicated that about 35% of the polymer was lost after heating to 600°C [Fig. 1(B)]. In the case of polybenzidine, about 45% was lost by heating to 600°C under the same conditions [Fig. 1(C)]. Thermal analyses carried out with other polymers showed a similar trend with respect to the amount of residual material after heating to 1000°C in a stream of nitrogen gas. Because of the high metal chelating properties of the polymer, it was suspected that the polymers may contain significant amount of metal complexed with the polymer and might have resulted in significant amount of residual materials after TGA. However, the elemental analyses of the polymers in a stream of oxygen at 980°C have in-

Table III. Elemental Analysis of Polymers Synthesized

Monomer (Ratio)	Polymer Composition (%)		
	Carbon	Hydrogen	Nitrogen
p-Phenylphenol/Aniline (1 : 1)	82.58(82.11)	5.51(6.51)	2.27(5.32)
p-Phenylphenol/Isoquinoline (1 : 1)	82.17(84.26)	4.85(5.72)	2.11(4.68)
p-Phenylphenol/Isoquinoline (1 : 2)	81.56(84.09)	4.94(5.64)	2.56(6.54)
p-Phenylphenol/8-Hydroxyquinoline (2 : 1)	81.64(81.63)	5.15(5.61)	2.19(2.88)
Benzidine	66.39(78.23)	5.53(6.56)	13.84(15.21)
1-Naphthol/Benzidine (1 : 1)	70.21(80.47)	4.94(6.14)	7.79(8.53)
1-Naphthol/Aniline (1 : 1)	71.58(80.99)	4.74(6.37)	4.35(5.90)
1-Naphthol/8-Hydroxyquinoline (1 : 1)	70.07(78.88)	4.73(5.23)	4.90(4.84)
2-Naphthol/Benzidine (1 : 1)	67.39(80.47)	5.18(6.14)	9.44(8.53)
2-Naphthol/8-Hydroxyquinoline (1 : 1)	61.39(78.88)	5.37(5.23)	8.99(4.84)
1,3-Naphthalenediol/Benzidine (1 : 1)	70.18(76.73)	4.42(5.85)	8.88(8.13)
1,5-Naphthalenediol/Benzidine (1 : 1)	65.50(76.73)	5.04(5.85)	10.80(8.13)

dicated no residual material (see Table III). The chemical and physical properties of this residual material have not been studied.

DSC thermograms (Fig. 2) of the polymers indicated branching and/or crosslinking after heating in an atmosphere of nitrogen gas as discussed later. DSC analysis of poly(*p*-phenylphenol) [Fig. 2(A)] indicated two exothermic heat flows, a minor one (1.14 J/g) at about 90°C and a major one (41.0 J/g) at about 200°C. The thermogram also indicated no well-defined melting point for the polymer. This finding was consistent with observations from melting point determinations with capillary tubes, namely no well defined melting point below 300°C. The two exothermic heat flows observed in the DSC thermogram of poly(*p*-phenylphenol) could be explained by a crystallization phase followed by branching and/or crosslinking.

That this exothermic reaction was not reversible was indicated by the DSC analysis of the heat treated polymer in an atmosphere of nitrogen gas. The DSC analysis has indicated the absence of any heat flow from the heat treated polymer. DSC analysis of poly(*p*-phenylphenol) chemically treated with epichlorohydrin [Fig. 2(B)] showed two peaks: the first peak was due to an endothermic reaction at 112.5°C and the second peak was due to an exothermic reaction at 204°C. DSC analysis of polybenzidine [Fig. 2(C)] indicated a well-defined melting point at about 210°C (a minor one with 5.10 J/g) followed by two exothermic reactions at 279 and 290°C. The DSC analysis of the benzidine polymer heat treated (at 300°C) in an atmosphere of nitrogen gas showed the lack of any heat exotherms from the polymer, indicating apparent branching/crosslinking of the polymer during the heat treatment.

In general, the polymers synthesized were not soluble in a number of organic solvents, solvent mixtures, or acids in the presence of lithium or sodium halides. This lack of solubility could be due to the high degree of aromaticity of the polymer with branching and/or to crosslinking of the polymer. The following solvents were studied to determine the solubility of the polymers synthesized: acetone, acetonitrile, *m*-cresol, dimethylformamide, dimethylsulfoxide, dioxane, ethyl alcohol, methyl alcohol, methylene chloride, tetrahydrofuran, and trifluroethanol. In addition, formic acid, acetic acid, and sulfuric acid were studied for solubilization of the polymers. Lithium chloride, lithium bromide, and sodium chloride were incorporated into the organic solvent or solvent mixture at a concentration of up to 3% (W/V) to study the solubility of the polymers in these salt–solvent mixtures. The solubility of the polymers was studied at 20, 40, and 60°C at a concentration of 0.1% (W/V). Of the solvent or solvent mixtures evaluated, only *m*-cresol and a solvent mixture of dimethylformamide/methanol (DMF : MeOH in the ratio of 4 : 1, V/V) solubilized poly(*p*-phenylphenol) at a concentration of 0.1% (W/V) at room temperature.

Column chromatography of poly(*p*-phenylphenol) solubilized in DMF : MeOH (4 : 1) indicated that the polymer had two well-separated peaks and the apparent molecular weight distribution ranged from 8000 to more than 400,000 using polystyrene as the reference standard. Because the polymer has an aromatic backbone with some degree of branching and crosslinking, the hydrodynamic volume and the

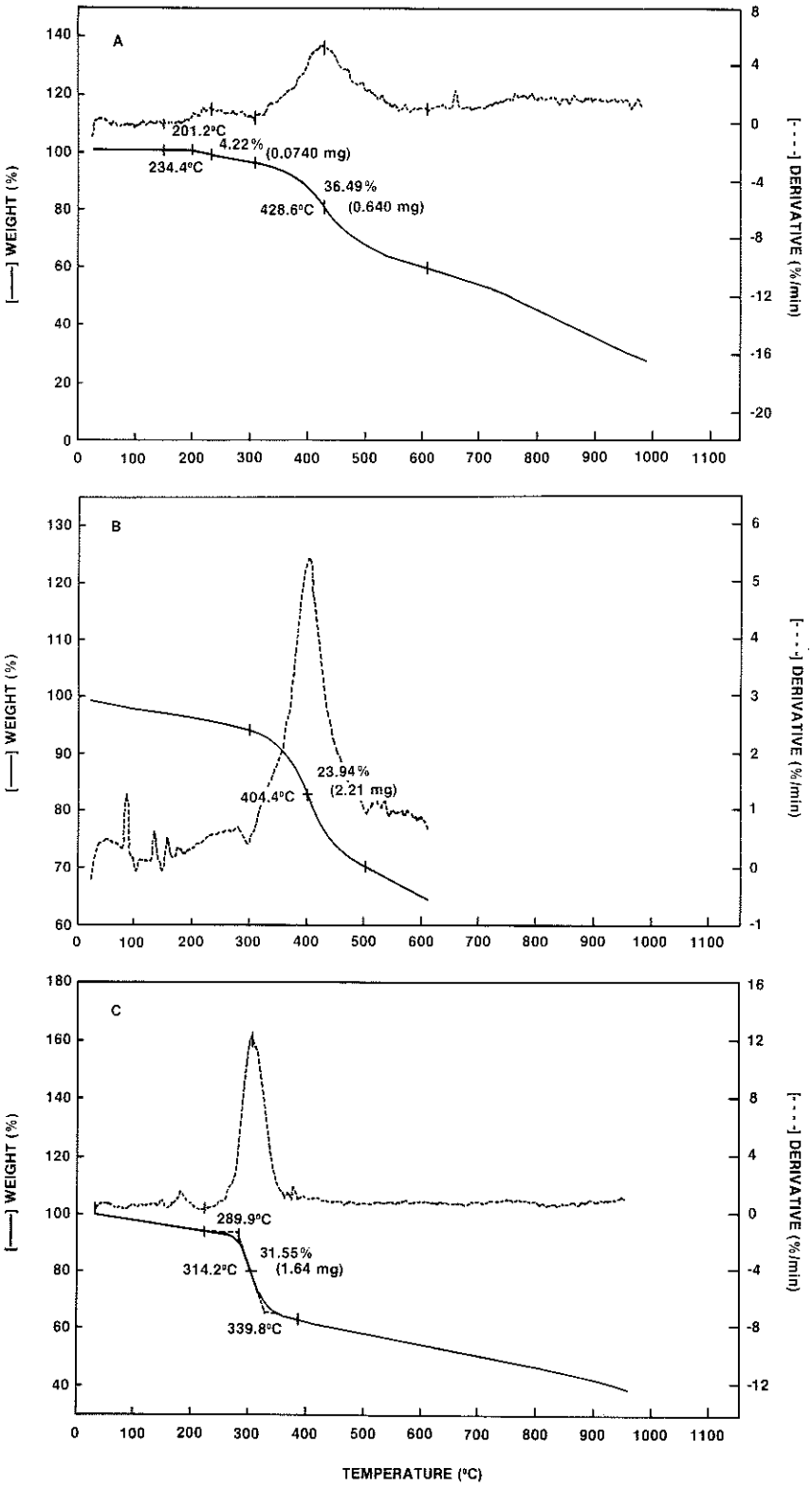


Figure 1. Thermogravimetric analysis of poly(*p*-phenylphenol) and polybenzidine. TGA of: (A) poly(*p*-phenylphenol), (B) poly(*p*-phenylphenol) chemically modified with epichlorohydrin, (C) polybenzidine.

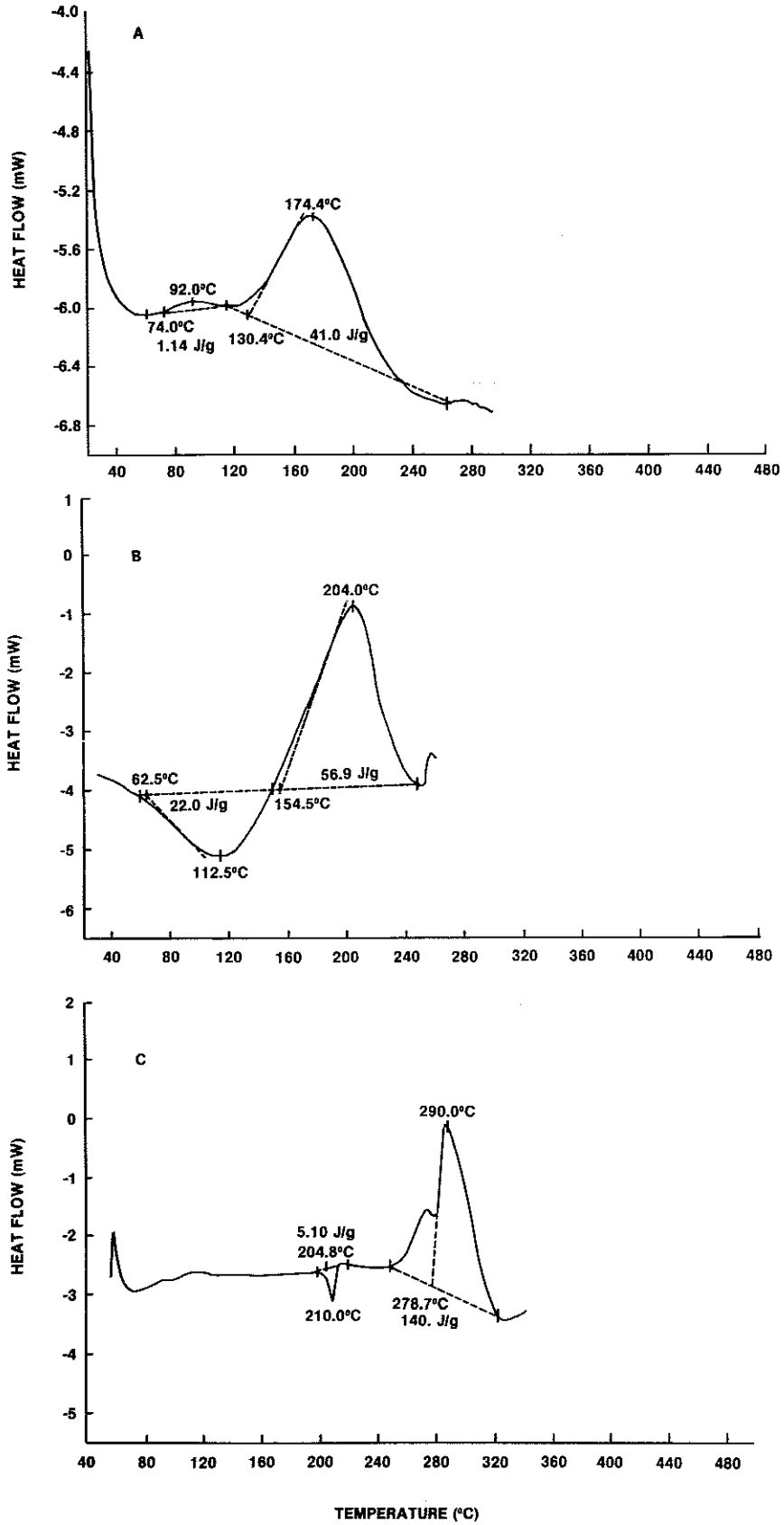


Figure 2. Differential scanning calorimetry of poly(*p*-phenylphenol) and polybenzidine. DSC of: (A) poly(*p*-phenylphenol), (B) poly(*p*-phenylphenol) chemically modified with epichlorohydrin, (C) polybenzidine.

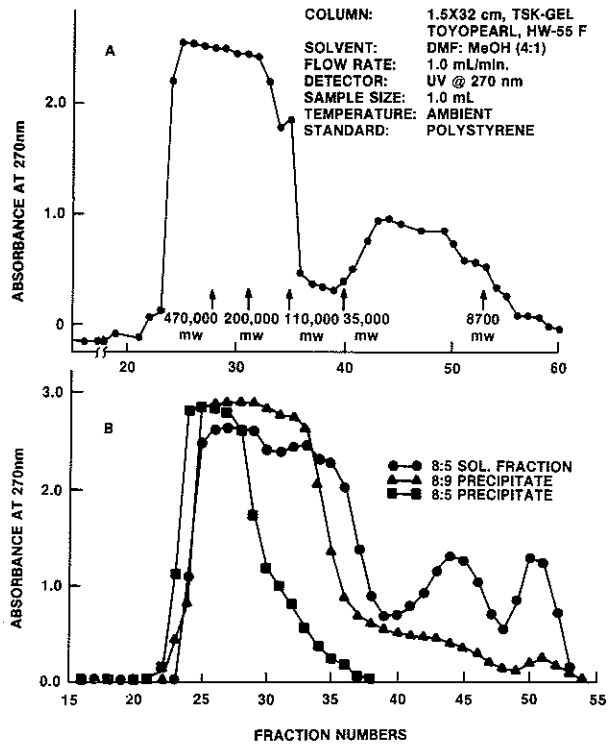


Figure 3. Column chromatography of poly(*p*-phenylphenol) synthesized. Chromatographic elution pattern of: (A) poly(*p*-phenylphenol) before fractionation, (B) fractions of poly(*p*-phenylphenol) fractionated by differential solubility of the polymer in solvents.

apparent molecular weight realized could be quite different from that of polystyrene standards. Conditions for the chromatographic analyses of the polymer are given in Figure 3(A).

The mechanism for the catalytic polymerization of phenols and aromatic amines by peroxidases has been described.[19] According to this mechanism, the phenolic free radicals formed by the enzymatic reaction are incorporated into a growing polymer chain. However this mechanism does not explain the bimodal distribution pattern observed with poly(*p*-phenylphenol) [see Fig. 3(A)]. A bimodal gel permeation chromatography (GPC) elution pattern, similar to the one observed with poly(*p*-phenylphenol), was reported earlier with polyaniline synthesized electrochemically under various reaction conditions.[20] These authors have given a number of possible causes for this bimodal GPC elution pattern, including polymer aggregation, chemical crosslinking, changes in the oxidation state, and existence of different mechanisms of polymerization.

Dordick et al.[3] have reported a molecular weight up to 26,000 for poly(*p*-phenylphenol) synthesized by horseradish peroxidase in dioxane solvent with acetate buffer and using viscometric techniques to determine the molecular weight. A decrease in molecular weight from a maximum of 26,000 by increasing the dioxane concentration above 85% in the reaction mixture, was shown by the authors. They also reported that the polymers synthesized with 80–90% dioxane in the reaction mixture were not completely soluble in dimethylformamide. The decrease in molecular weight reported could be explained by the presence of high molecular weight insoluble polymer excluded from the molecular weight determinations.

To determine the physical and chemical properties of poly(*p*-phenylphenol) with respect to its molecular weight, the polymer synthesized was separated into well-defined fractions by differential solubility of the polymer in solvent mixtures of DMF : MeOH at different ratios. The apparent molecular weights of these polymer fractions were determined using TSK-GEL Toyopearl packed columns described above. The elution patterns of these fractions are shown in Figure 3(B). The 8 : 5 (DMF: MeOH) precipitate fraction was primarily of high molecular weight, whereas the 8 : 5 (DMF : MeOH) soluble fraction contained poly(*p*-phenylphenol) with a broad range of molecular weights (from less than 1000 to greater than 400,000), as observed in the parent polymer [see Fig. 3(A)]. Based strictly on the solubility of the polymers in the DMF : MeOH (8 : 5) solvent mixture under the conditions used, it appears from these fractionation studies that there are at least two types of high molecular weight polymers in the parent polymer. The column chromatography indicated that the 8 : 9 (DMF : MeOH) precipitate contained mostly the high molecular weight fraction.

Thermal properties of the 8 : 5 (DMF : MeOH) poly(*p*-phenylphenol) precipitate fraction obtained above were determined by TGA and DSC analyses. The TGA thermogram of the 8 : 5 fraction indicates [Fig. 4(A)] that there are two inflection points, the first one at 340°C and the second one at 436°C, and about 55% of the polymer was lost due to evaporation and/or decomposition by heating to 600°C in an atmosphere of nitrogen gas. The DSC analysis of the 8 : 5 fraction [Fig. 4(B)] indicated a single exothermic peak at 297°C. DSC analysis of the heat treated 8 : 5 fraction (in an atmosphere of nitrogen) showed neither heat gain or loss from the polymer again indicating apparent branching and/or crosslinking of the polymer during the annealing step.

The FTIR spectrum of the poly(*p*-phenylphenol) [Fig. 5(A)] showed strong absorption peaks for the presence of multiple substitutions (up to four) on

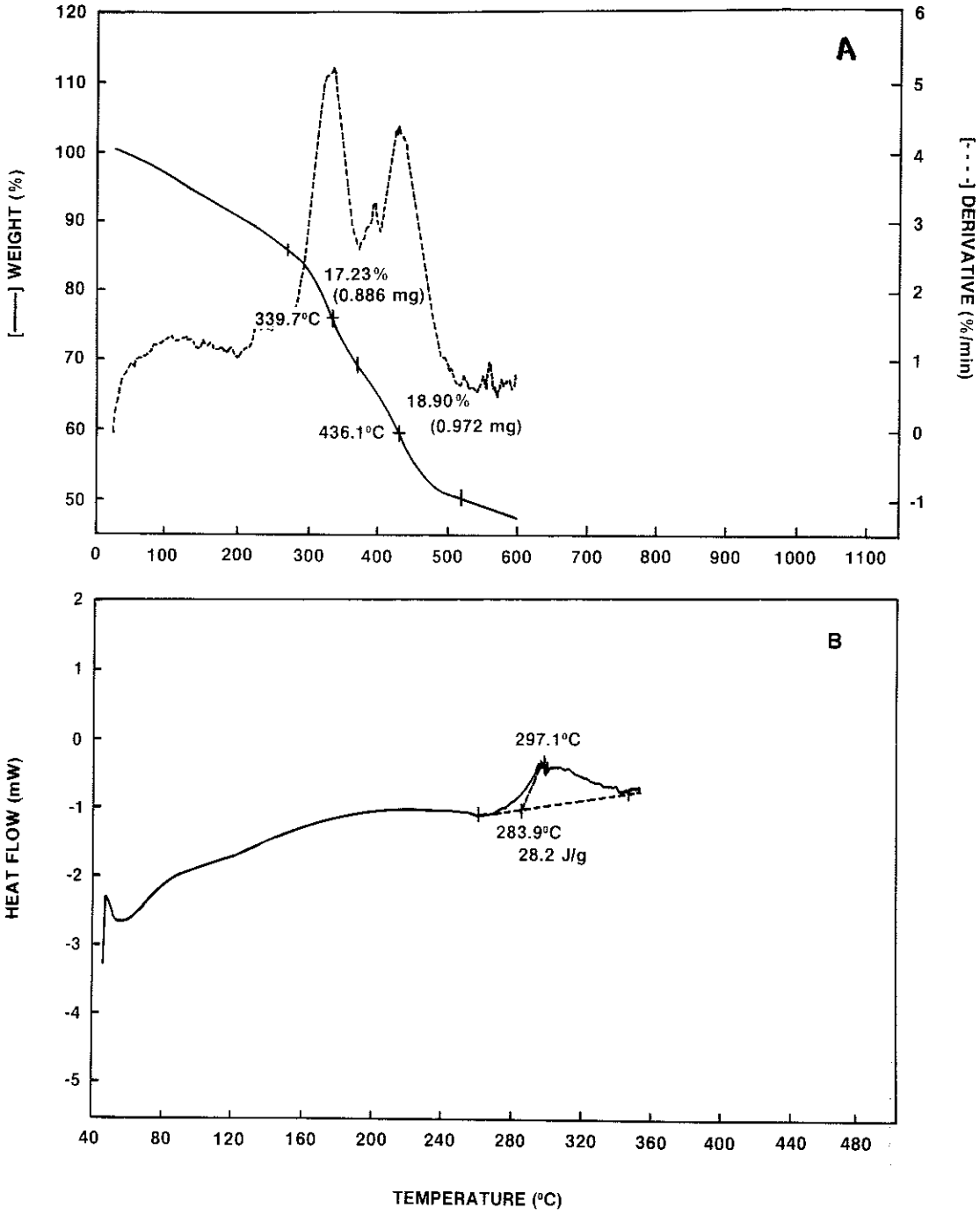


Figure 4. Thermograms of poly(*p*-phenylphenol) fractions: (A) thermogravimetric analysis of the 8 : 5 precipitate fraction of poly(*p*-phenylphenol), (B) differential scanning calorimetry of the 8 : 5 precipitate fraction of poly(*p*-phenylphenol).

the aromatic ring. A comparison of the polymer FTIR spectrum with that of the monomer (not shown) indicates that the monomer spectral peak at wavenumber 820 disappeared after polymerization [Figs. 5(A) and 5(C)]. The polymer spectra also indicate that the substitutions on the aromatic ring were at positions 1, 2, 4, and 6 with respect to phenolic—OH. Phenoxy ether linkages were not observed in the spectrum. FTIR analysis of polybenzidine [Fig. 5(B)] showed the presence of aromatic primary and secondary amines (wave number 3348) with minor peaks for the presence of the mul-

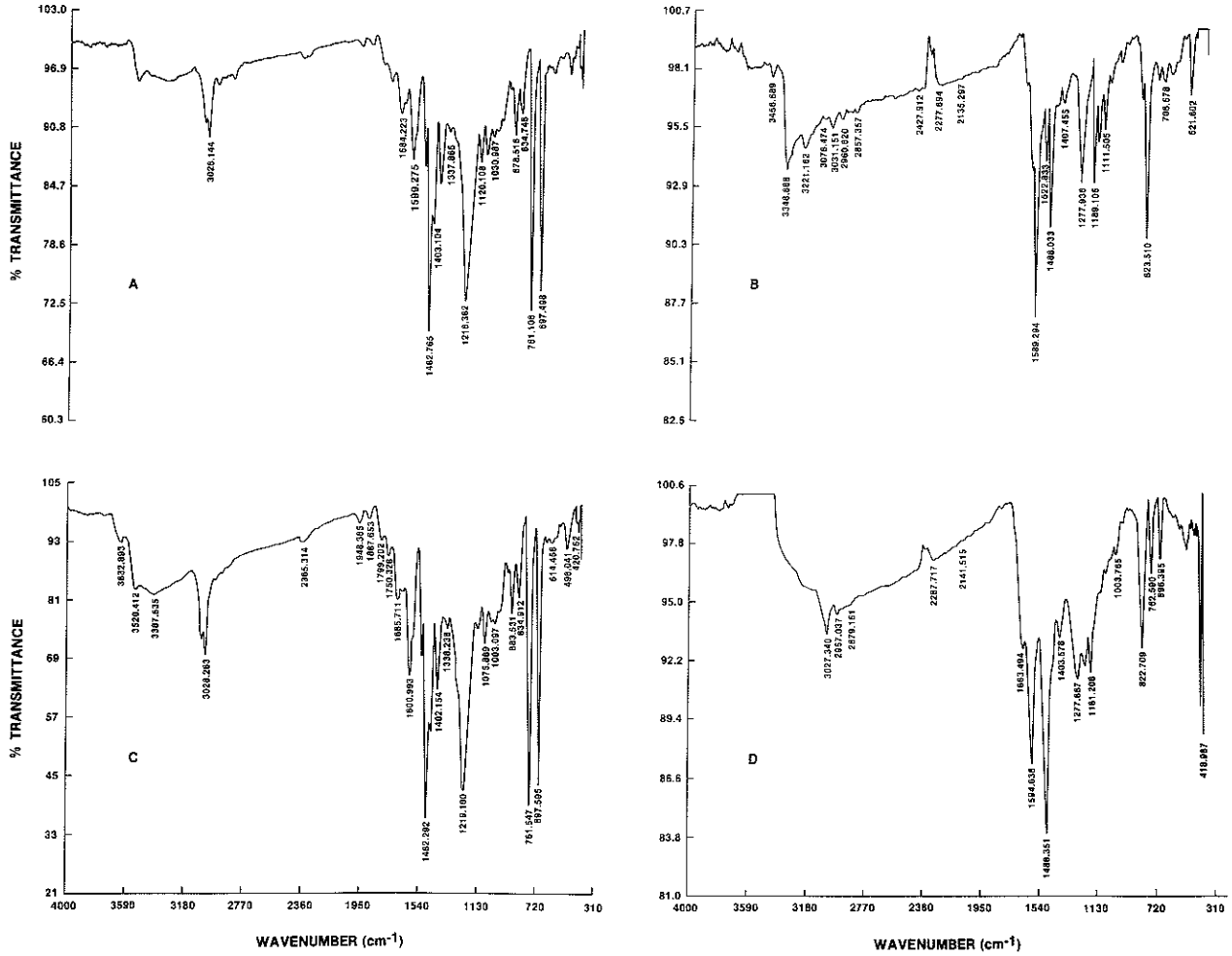


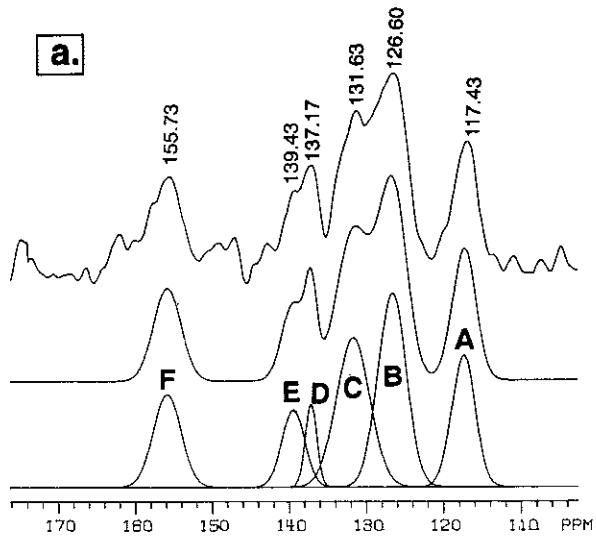
Figure 5. Fourier transform infrared spectra of polymers synthesized. FTIR spectrum: (A) of poly(*p*-phenylphenol), (B) polybenzidine, (C) poly(*p*-phenylphenol) chemically modified with epichlorohydrin, (D) copolymer (*p*-phenylphenol)–benzidine (1 : 1).

tisubstituted aromatic ring. The FTIR spectrum of poly(*p*-phenylphenol) chemically modified with epichlorohydrin [Fig. 5(C)] was essentially the same as that obtained with unmodified poly(*p*-phenylphenol). The FTIR spectrum of the copolymer (*p*-phenylphenol-benzidine) (1 : 1) [Fig. 5(D)] contained all the major spectral peaks of the two homopolymers.

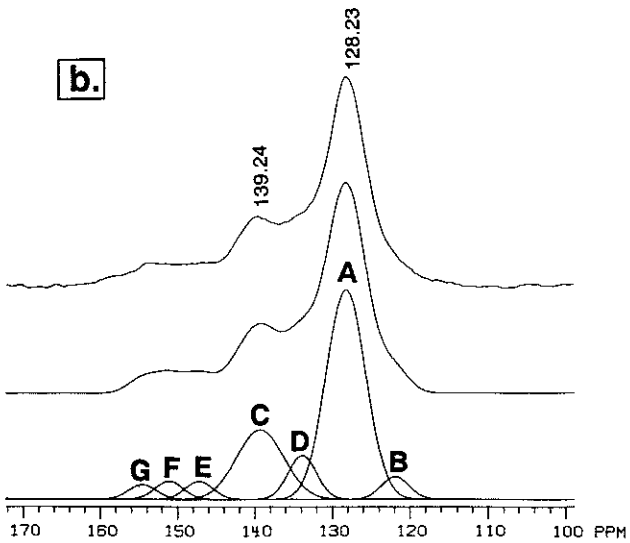
Figure 6 shows the ^{13}C solid state CP/MASS NMR spectra of *p*-phenylphenol [Fig. 6(a)] and poly(*p*-phenylphenol) [Fig. 6(b)]. Spinning side bands present in the spectra of poly(*p*-phenylphenol) and polybenzidine (by the presence of the large chemical shift anisotrophy of aromatic carbons), are not shown in the Figures 6 and 7. In general, all the aromatic signals were much broader in the polymer. The monomer signal at 117.43 ppm was shifted down field to 128.23 ppm with ortho substitutions in the aromatic ring. This major NMR signal with the polymer (55.48%) shows that an ortho-substituted product is the major constituent of poly(*p*-phenylphenol) synthesized [see Figs. 8(a) and 8(b)]. Earlier ^{13}C-NMR (with cross-polarization, magic-angle sample spinning) studies with chemically synthesized poly(*p*-phenylene) had shown a para-linked structure for the polymer.[21]

Figure 7 shows the NMR spectra of benzidine and polybenzidine. Figures 7(a) and 7(b) show the ^{13}C solid-state CP/MASS NMR spectra of benzidine and polybenzidine, respectively. Proton NMR spectrum of polybenzidine in deuterated dimethylsulfoxide (DMSO-d_6) solution is given in Figure 7(c).

The solid-state NMR spectrum of polybenzidine indicates (127 and 142.37 ppm) that the polymerization is through *ortho*-carbon–nitrogen and *ortho*-carbon–carbon linkages [see Figs. 9(A), 9(B), and



LINE	PPM	AREA	% AREA
A	117.43	768	16.34
B	126.60	1359	28.92
C	131.63	1242	26.43
D	137.17	243	5.17
E	139.43	437	9.30
F	155.73	649	13.81

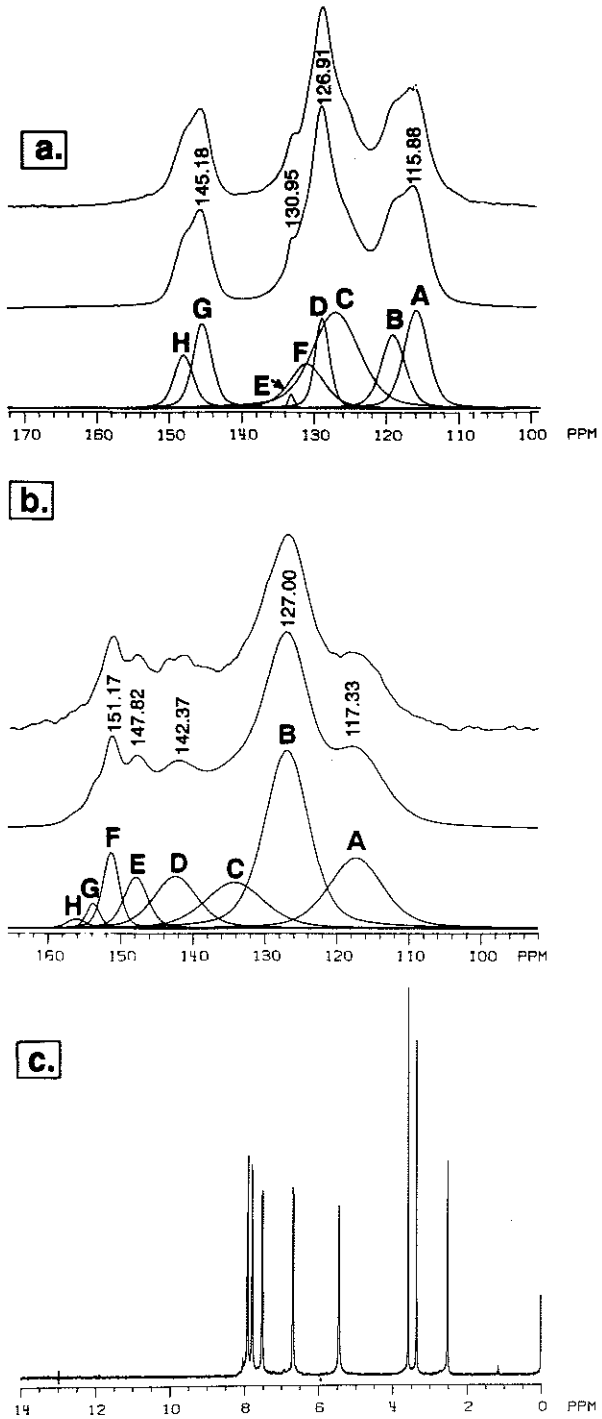


LINE	PPM	AREA	% AREA
A	128.23	1380	55.48
B	121.92	106	4.26
C	139.24	549	22.03
D	133.79	210	8.44
E	147.13	86	3.45
F	151.01	86	3.45
G	154.48	71	2.85

Figure 6. ^{13}C Solid-state NMR spectra of (a) *p*-phenylphenol and (b) poly(*p*-phenylphenol).

9(C)]. Solid-state ^{13}C-NMR spectra of polyaniline prepared by oxidizing aniline with ammonium peroxydisulfate, showed three broad resonance at 124.5, 140, and 158 ppm indicating the existence of many resonances with localized double bonds in an alternating benzoid-quinoid structure linked with an imine at para positions.[22] Proton NMR of polybenzidine in DMSO-d_6 solution shows multiple signals for aromatic and amine protons. The presence of four doublet aromatic proton peaks (7.91/7.887, 7.783/7.760, 7.518/7.495, and 6.684/6.662) were due to coupling between the nearby non-equivalent protons. The ortho aromatic hydrogen was shifted upfield to 6.67 by the amine group on the ring. This spectral line indicates the presence of unsubstituted *ortho* positions in the polymer (see Fig. 9). The peak at 5.43 ppm is due the presence of a secondary amine proton [see Fig. 9(A)]. The other two peaks (at 3.34 ppm and 3.57 ppm) could be assigned to amine protons [see Figs. 9(A), 9(B), and 9(C)]. The presence of amine protons in the polymer was confirmed by D_2O exchange studies.

Solubility studies, molecular weight determinations, and thermal, FTIR and NMR analyses indicate that the polymer formation in these enzyme catalyzed reactions in solvents is through the ortho substitutions, and to a less extent through para substitution on the aromatic ring. This type of condensation reaction could result in a polymer with highly conjugated aromatic backbone with a planar struc-



LINE	PPM	AREA	% AREA
A	115.88	553	16.63
B	119.04	420	12.50
C	126.91	1079	32.11
D	128.70	307	9.13
E	133.01	19	0.56
F	130.95	361	10.74
G	145.18	374	11.13
H	147.81	241	7.17

LINE	PPM	AREA	% AREA
A	117.33	799	19.55
B	127.00	1634	39.99
C	134.10	563	13.77
D	142.37	472	11.55
E	147.82	251	6.14
F	151.17	260	6.36
G	153.66	60	1.66
H	155.99	39	0.95

Figure 7. NMR spectra of benzidine and polybenzidine: (a) ^{13}C solid-state NMR spectrum of benzidine, (b) ^{13}C solid state NMR spectrum of polybenzidine, (c) ^{1}H-NMR spectrum of polybenzidine in solution.

ture. Based on these findings the possible reaction sequences and types of polymers formed by the enzyme catalyzed polymerization of *p*-phenylphenol and benzidine are given in Figures 8 and 9, respectively.

CONCLUSIONS

A number of homopolymers and copolymers were synthesized from substituted phenolic and aromatic amines by horseradish peroxidase catalyzed reac-

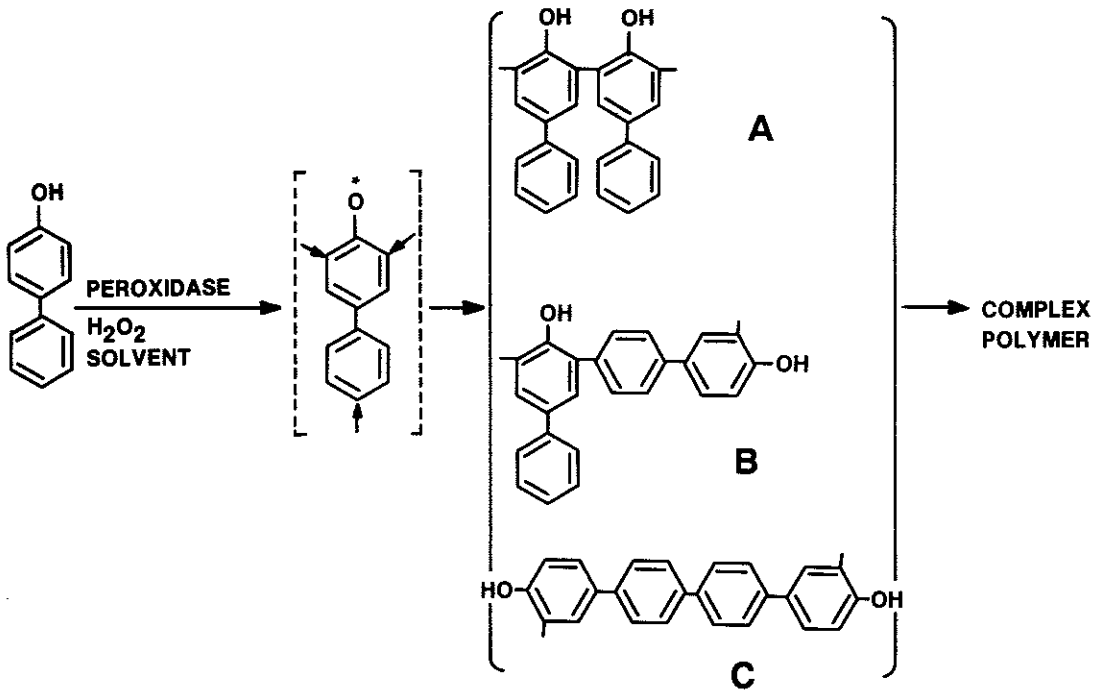


Figure 8. Predicted reaction mechanism and different types of *p*-phenylphenol polymers formed.

tions in solvents containing buffers at pH values 5.0, 7.0, and 7.5. Increasing the concentration of the solvent in the reaction mixture decreased the yield of the polymer but increased the melting point of the polymer synthesized. Elemental analysis of the polymers synthesized indicated that the incorpo-

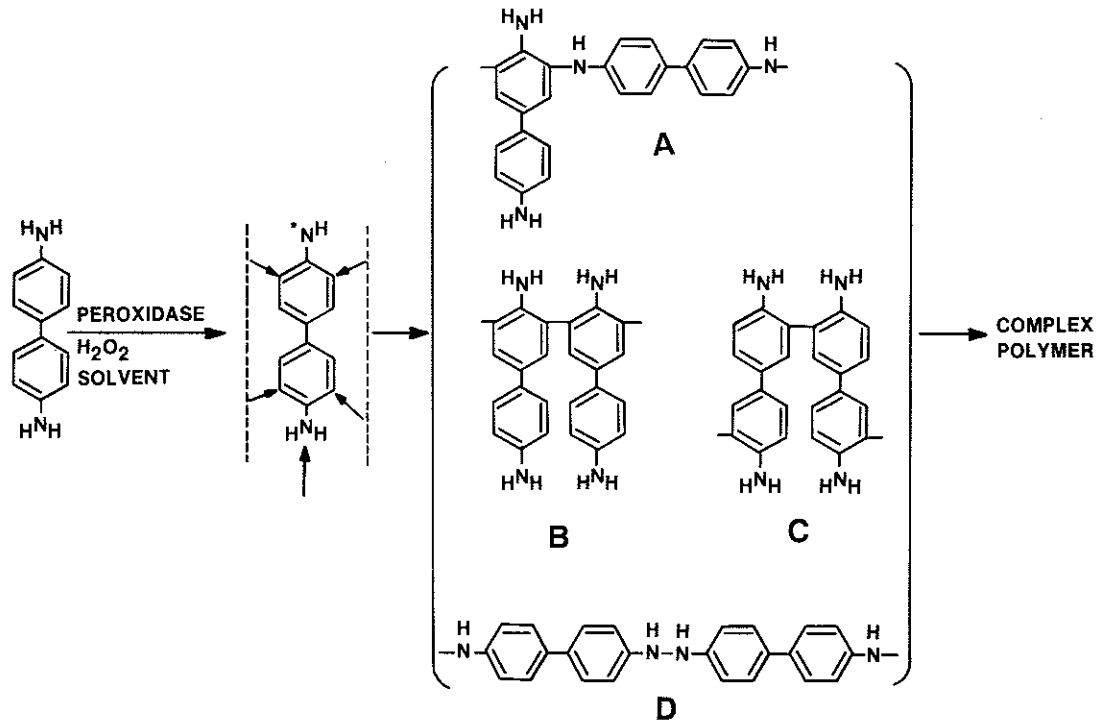


Figure 9. Predicted reaction mechanism and different types of benzidine polymers formed.

ration of nitrogen containing monomers into the polymer varied from 45 to 100% and the amount of incorporation was based on the concentration and chemical nature of the monomer used in the reaction mixture. This is of critical importance for the controlled incorporation of monomers with specific functional groups into polymers with desired functional properties by enzyme coupled reactions in solvents.

Thermal analyses showed high melting points and temperature resistance of the polymers synthesized. About 30–40% of the polymer was not destroyed by heating up to 1000°C in a stream of nitrogen gas. Branching/crosslinking was also observed upon heating the polymers. The polymers synthesized had very low solubility in a number of solvents or solvent mixtures. Poly(*p*-phenylphenol) was partially fractionated by differential solubility of the polymer in a solvent mixture of DMF : MeOH. The highest apparent molecular weight of the fractions of this polymer was greater than 400,000 using polystyrene reference standards.

FTIR and NMR spectra of the polymers synthesized indicated that the polymerization by this enzyme catalyzed reaction is by ortho substitution on the ring with less than 10% of the substitution on the para position. This type of condensation polymer product could result in planar and rigid structures. Low solubility, high melting point, and thermal properties of these polymers are indicative of the wholly aromatic structure.

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